Leishmania mexicana Centrin Knockout Parasites Promote M1-polarizing Metabolic Changes

Volpedo, Greta, Ohio State University; Pacheco-Fernandez, Thalia, Ohio State University; Oljuskin, Timur, USDA; Azodi, Nazli, FDA/CBER/OBRR; Markle, Hannah, FDA/CBER/OBRR; Hamano, Shinjiro, Nagasaki University; Matlashewski, Greg, McGill University; Satoskar, Abhay R., Ohio State University; Gannavaram, Sreenivas, FDA/CBER/OBRR; Nakhasi, Hira, FDA/CBER/OBRR

Abstract

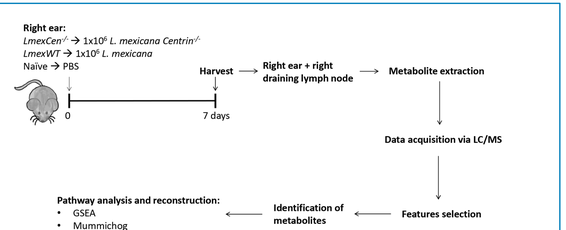
Leishmaniasis is a protozoan disease with no vaccine currently approved. Our group has developed genetically modified *centrin*-deficient *Leishmania* parasites (*LmexCen*$^{-/-}$) that show excellent safety and efficacy in pre-clinical studies. *LmexCen*$^{-/-}$ parasites have been shown to induce a Th1 response and reduce Th2 activation, contrary to wild type *L. mexicana (Lmex*WT*)*. We applied untargeted mass spectrometric analysis to both parasite strains which showed enriched pentose phosphate pathway (PPP) in ears immunized with *LmexCen*$^{-/-}$ compared to naïve and *Lmex*WT groups. This pathway promotes an M1 phenotype in macrophages, suggesting a switch to a pro-inflammatory phenotype following *LmexCen*$^{-/-}$ inoculation. PPP inhibition in macrophages cultured with *LmexCen*$^{-/-}$ led to diminished nitric oxide, IL-12, and IL-1β production, hallmarks of classical PPP activation. Overall, our study revealed novel immune regulatory mechanisms that may be critical for the induction of protective immunity.

Introduction

Understanding early immunoregulatory mechanisms following vaccination with *LmexCen*$^{-/-}$ parasites is important to evaluate safety and efficacy, and metabolomic reprogramming of host cells drives such early changes. A growing body of work shows that metabolic changes in macrophages infected with *Leishmania* can impact the availability of nutrients necessary for replication and affect the cell's polarization and host potential[1]. For instance, M1 macrophages in *Leishmania* dermal granulomas upregulate the PPP, important for reactive oxygen species (ROS) production[1] and M1 effector responses[2]. Similarly, ROS and nitric oxide (NO) release are markers of PPP activity in *Trypanosoma cruzi* infections[3]. Interestingly, upregulation of the PPP in macrophages occurs within one hour of lipopolysaccharide (LPS) stimulation, and several hours prior to the induction of pro-inflammatory mediators such as superoxide, IL-1β, TNF-α, IL-6, and IL-12[4]. In this study, we further investigated metabolic drivers of immunological changes associated with the previously shown enhanced Th1 responses in *LmexCen*$^{-/-}$[5].

Materials and Methods

C57/BL6 mice were infected with *LmexWT* and *LmexCen*$^{-/-}$. Infected ear tissues were collected 7 days post infection and analyzed by untargeted LC/MS mass spectrometry. Data were analyzed with the Metaboanalyst 5.0 for pathway analysis and Metscape 3.1.1 for integrative network analysis. To verify, murine bone marrow-derived macrophages (BMDMs) were infected with *LmexWT* and *LmexCen*$^{-/-}$ and cultured with PPP inhibitors. Gene expression levels of interest were measured via qRT-PCR.



Experimental design for metabolomic analysis of infection with *Lmex*WT and immunization with *LmexCen*$^{-/-}$. Female C57BL/6 mice were injected intradermally in the ear with stationary phase *Lmex*WT, *LmexCen*$^{-/-}$ parasites, or PBS. After 7 days, the injected ears and their respective draining lymph nodes were collected and processed for mass spectrometry. The data acquired was used to identify enriched metabolites and determine activated pathways in the tissue. N=5 for each group. Abbreviations: LC/MS: liquid chromatography / mass spectrometry; GSEA: gene set enrichment analysis.

Results and Discussion

Our results showed that the PPP was enriched in the ears of *LmexCen*$^{-/-}$-immunized mice, compared to naïve and *LmexWT*-infected mice. Mass spectrometry analysis revealed upregulated PPP in the *LmexCen*$^{-/-}$ group, which is known to polarize macrophages towards a proinflammatory M1 phenotype. Treatment with PPP inhibitors resulted in a significant reduction in nitric oxide (NO) levels, IL-12 levels in both the uninfected and *LmexCen*$^{-/-}$ groups compared to their controls. Similar reduction in IL-1β was also observed in the *LmexCen*$^{-/-}$ group compared to controls.

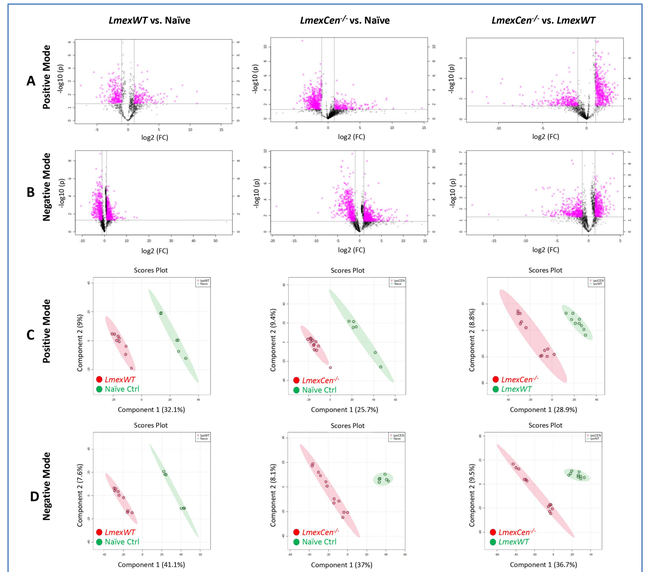


Figure 1. Infection with *Lmex*WT and immunization with *LmexCen*$^{-/-}$ display different metabolic signatures in at the inoculation site. Normalized data from ear tissue from C57BL/6 mice after 7 days of infection with *Lmex*WT, immunization with *LmexCen*$^{-/-}$, or naïve control was used to perform statistical analysis. **A, B)** Features selected by volcano plot from positive (**A**) and negative (**B**) modes for ear tissue of *Lmex*WT vs. naïve, *LmexCen*$^{-/-}$ vs. naïve, and *LmexCen*$^{-/-}$ vs. *Lmex*WT mice using LC/MS with fold change threshold (x) 2 and t-tests threshold (y) 0.05. Both fold changes and p-values are log transformed. **C, D)** Partial least squares-discriminant analysis (PLS-DA) from positive (**C**) and negative (**D**) mode for ear tissue of *Lmex*WT vs. naïve, *LmexCen*$^{-/-}$ vs. naïve, and *LmexCen*$^{-/-}$ vs. *Lmex*WT mice.

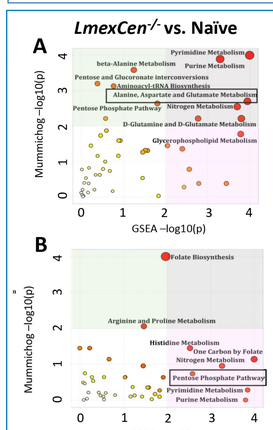


Figure 2. Metabolic pathways enriched in mice immunized with LmexCen-/- at the inoculation site. Normalized data from ear tissue from C57BL/6 mice after 7 of immunization with LmexCen-/- or naïve control was used to perform peaks to pathway analysis. Using the MS Peaks to Paths module in MetaboAnalyst5.0, the mummichog and gene set enrichment analysis (GSEA) p-values were combined. The Integrated MS Peaks to Paths plot summarizes the results of the Fisher's method for combining mummichog (y) and GSEA (x) p-values from the positive (A) negative (B) mode data sets, indicating the metabolic pathways enriched. The size and color of the circles correspond to their transformed combined p-values. Large and red circles are considered the most perturbed pathways. The colored areas show the significant pathways based on either mummichog (blue) or GSEA (pink), or the purple area highlights significant pathways identified by both algorithms. Highlighted by the black boxes are pathways associated with a protective immune response against leishmaniasis.

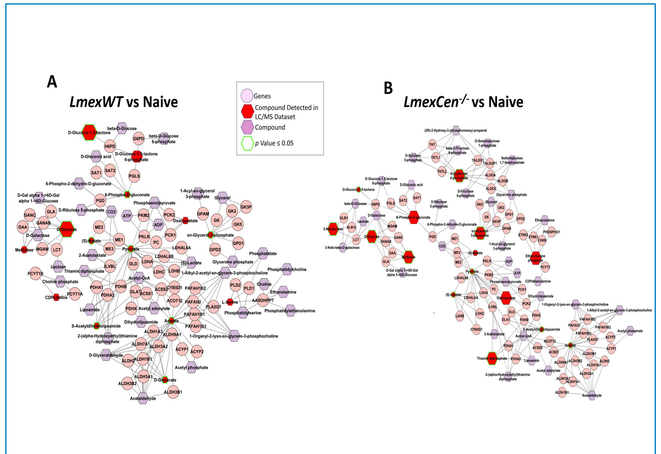


Figure 3. Immunization with LmexCen-/- leads to enriched Pentose Phosphate Pathway metabolism. Normalized data from ear tissue from C57BL/6 mice after 7 days of infection with LmexWT vs naïve control (A) or immunization with LmexCen-/- versus naïve control (B) was used to perform statistical analysis. Metabolite network identified with Metscape. Metabolites of the Pentose Phosphate Pathway are represented in this graph. Larger hexagons represent up-regulation, while smaller hexagons represent down-regulation. Red hexagons represent compounds detected in the data set, while hexagons with a green outline represent statistically significant metabolites (p-value ≤ 0.05). The purple hexagons represent compounds that are associated with the pathway but are not detected in the input dataset. The pink circles represent the genes regulating the biosynthetic activities.

Dataset	Compound Name	HMDB ID	P. value	Log FC
LmexCEN$^{-/-}$ vs Naïve	D-Gluconolactone	HMDB0000150	0.07614	0.469463
LmexCEN -/- vs Naïve	D-Gluconolactone	HMDB0000150	0.00323	Infinity
LmexCEN -/- vs Naïve	D-Gluconolactone	HMDB0000150	0.06476	0.438271
LmexCEN -/- vs Naïve	D-Gluconolactone	HMDB0000150	0.03838	-0.83846
LmexCEN -/- vs Naïve	2-deoxy-D-ribose-5-phosphate	HMDB0001031	0.05156	0.430808
LmexCEN -/- vs Naïve	fructose-6-phosphate	HMDB0000124	0.01374	0.859422
LmexCEN -/- vs Naïve	fructose-6-phosphate	HMDB0000124	0.013	0.374674
LmexCEN -/- vs Naïve	fructose-6-phosphate	HMDB0000124	0.11882	Infinity
LmexCEN -/- vs Naïve	fructose-6-phosphate	HMDB0000124	0.04761	0.097545
LmexWTvsLmexCEN -/-	D-Gluconolactone	HMDB0000150	0.37318	-0.16859
LmexWTvsLmexCEN -/-	D-Gluconolactone	HMDB0000150	0.02529	-0.21483
LmexWTvsLmexCEN -/-	2-deoxy-D-ribose-5-phosphate	HMDB0001031	0.24624	-0.19461
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.00016	-0.22084
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.00478	-0.70155
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.00537	-0.57994
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.15585	-0.65417
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.08191	0.145185
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.01194	-0.75432
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.27226	-0.22221
LmexWTvsLmexCEN -/-	fructose-6-phosphate	HMDB0000124	0.14201	-0.74394
LmexWTvs Naive	D-Gluconolactone	HMDB0000150	0.0207	Inifinity
LmexWTvs Naïve	D-Gluconolactone	HMDB0000150	0.04355	0.315925
LmexWTvs Naïve	fructose-6-phosphate	HMDB0000124	0.00375	0.211366
LmexWTvs Naïve	fructose-6-phosphate	HMDB0000124	0.0097	0.408554
LmexWTvs Naïve	fructose-6-phosphate	HMDB0000124	0.01975	-0.54961
LmexWTvs Naïve	fructose-6-phosphate	HMDB0000124	0.02603	-0.1233
LmexWTvs Naïve	fructose-6-phosphate	HMDB0000124	0.55928	0.180685

Table 1. Pentose phosphate pathway mediators enriched in the *LmexCen-/-* and *Lmex*WT datasets. Normalized data ear tissue from C57BL/6 mice inoculated with *LmexCen-/-, Lmex*WT or naïve controls was used to identify pentose phosphate pathway metabolites.

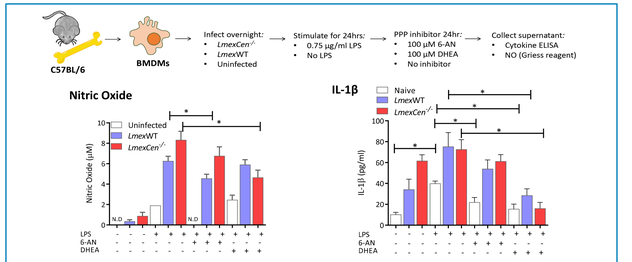


Figure 4. Pentose phosphate pathway-dependent nitric oxide and IL-1β release in BMDMs. BMDMs were extracted from C57BL/6 mice and cultured with medium, *Lmex*WT, or *LmexCen-/-* and stimulated in vitro by LPS for 24 hrs, and 6-AN or DHEA for 24 hrs. Nitric oxide production was determined by Griess reaction. IL-1β production was determined by cytokine ELISA. Data represents one of three experiments with N=3-5 per group. *P < 0.05, ** P ≤ 0.01 and *** P ≤ 0.001 unpaired t test. Error bars represent SEM. Abbreviations: LPS, lipopolysaccharide; 6-AN, 6-aminonicotinamide; DHEA, dehydroepiandrosterone.

Conclusion

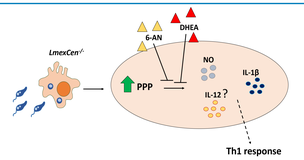


Figure 5. Immunization with *LmexCen*$^{-/-}$ parasites leads to induction of pentose phosphate pathway. Graphical scheme of *LmexCen*$^{-/-}$-induction of the PPP, resulting in production of NO, IL-12, and IL-1b in macrophages. Abbreviations: 6-AN, 6-aminonicotinamide; DHEA, dehydroepiandrosterone; NO, nitric oxide.

Application of metabolomic analysis to vaccine studies identified mechanisms of immune protection and may help identify novel biomarkers of vaccine efficacy of a live-attenuated vaccine candidate for human leishmaniasis. Our results are interesting given *Lmex*WT parasites are known to polarize macrophages towards an M2 anti-inflammatory phenotype[6]. In a parallel study performed in *L. major* WT versus *L. major Cen*$^{-/-}$, the prominently impacted pathways differed from that of the *L. mexicana* studies presented here, underlying the distinct role of metabolomic reprogramming in vaccination immunity versus pathogenicity induced by different strains of *Leishmania* infection.

References

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